

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/EP05/050899

International filing date: 01 March 2005 (01.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: EP
Number: 04405123.3
Filing date: 02 March 2004 (02.03.2004)

Date of receipt at the International Bureau: 08 April 2005 (08.04.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse



**Europäisches
Patentamt**

**European
Patent Office**

**Office européen
des brevets**

Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

04405123.3

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



Anmeldung Nr:
Application no.: 04405123.3
Demande no:

Anmeldetag:
Date of filing: 02.03.04
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

EPFL Ecole Polytechnique Fédérale
de Lausanne
CM - Ecublens
1015 Lausanne
SUISSE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se référer à la description.)

Mutants of 06-Alkylguanine-DNA Alkyltransferase

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s)
revendiquée(s)

Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

G12N9/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PL PT RO SE SI SK TR LI

Mutants of O⁶-Alkylguanine-DNA Alkyltransferase

Field of the Invention

5 The present invention relates to mutants of wild type human O⁶-alkylguanine-DNA alkyltransferase (hAGT) and to methods of transferring a label from substrates to fusion proteins consisting of these O⁶-alkylguanine-DNA alkyltransferase mutants and proteins of interest.

10 Background of the invention

The mutagenic and carcinogenic effects of electrophiles such as N-methyl-N-nitrosourea are mainly due to the O⁶-alkylation of guanine in DNA. To protect themselves against DNA-alkylation, mammals and bacteria possess a protein, O⁶-alkylguanine-DNA alkyltransferase (AGT) which repairs these lesions. AGT transfers the alkyl group from the position O-6 of alkylated guanine and guanine derivatives to the mercapto group of one of its own cysteines, resulting in an irreversibly-alkylated-AGT. The underlying mechanism is a nucleophilic reaction of the S_N2 type which explains why not only methyl groups, but also benzylic groups are easily transferred. As overexpression of human AGT (hAGT, SEQ ID NO:1) in tumour cells is the main reason for resistance to alkylating drugs such as procarbazine, dacarbazine, temozolomide and bis-2-chloroethyl-N-nitrosourea, inhibitors of AGT have been proposed for use as sensitisers in chemotherapy (Pegg *et al.*, Prog Nucleic Acid Res Mol Biol 51:167-223, 1995). US 5,691,307 describes O⁶-benzylguanines carrying various substituents in the benzyl group, and their use for depleting AGT levels in tumor cells and thereby increasing responsiveness to alkylating anti-tumor drugs. Likewise, WO 97/20843 discloses further AGT depleting compounds representing O⁶-benzyl- and O⁶-heteroarylmethyl-pyrimidine derivatives.

DE 199 03 895 discloses an assay for measuring levels of AGT which relies on the reaction between biotinylated O⁶-alkylguanine derivatives and human AGT which leads to biotinylation of the AGT. This in turn allows the separation of the AGT on a streptavidin coated plate and its detection, e.g. in an ELISA assay. The assay is suggested for monitoring the level of AGT in tumour tissue and for use in screening for AGT inhibitors.

35 Damoiseaux *et al.*, ChemBiochem 4:285-287, 2001, disclose modified O⁶-alkylated guanine derivatives incorporated into oligodeoxyribonucleotides for use as chemical probes for

labelling human AGT, again to facilitate detecting the levels of this enzyme in cancer cells to aid in research and in chemotherapy.

5 WO 02/083937 discloses a method for detecting and/or manipulating a protein of interest wherein the protein is fused to AGT and the AGT fusion protein contacted with an AGT substrate carrying a label, and the AGT fusion protein detected and optionally further manipulated using the label. Several AGT fusion proteins to be used, general structural principles of the AGT substrate and a broad variety of labels and methods to detect the label useful in the method are described. Although other forms of AGT are mentioned, only human
10 AGT is exemplified.

PCT/EP03/10859 describes particular AGT fusion proteins to be used in the mentioned method for detecting and/or manipulating a protein of interest, labelled fusion proteins obtainable by this method, and the method using the particular AGT fusion proteins.
15

PCT/EP03/10889 discloses additional AGT substrates carrying a label particularly suitable in the mentioned method for detecting and/or manipulating a protein of interest, and the application of such particularly labelled substrates. This patent application also describes methods of manufacture of these additional AGT substrates.
20

Human AGT mutant Gly160Trp (Xu-Welliver *et al.*, Biochemical Pharmacology 58:1279-1285, 1999) is somewhat more reactive towards benzylguanine derivatives than wild type human AGT. Juillerat *et al.*, Chem Biol 10:313-317, 2003, prepared a number of mutants of human AGT in the search for more reactive partners for efficient *in vivo* labeling of AGT
25 fusion proteins with synthetic substrates. Mutations in position 140, 157, 159 and 160 were reported. The mutant Asn157Gly Ser159Glu shows increased activity against benzylguanine derivatives by a factor of approximately 20 compared to wild type hAGT.

The following additional mutations in hAGT have been shown to disrupt DNA binding of hAGT but do not significantly interfere with the activity against benzylguanine derivatives: Lys125Ala, Ala127Thr and Arg128Ala, see Lim *et al.*, EMBO J 15:4050-4060, 1996, and Daniels *et al.*, EMBO J 19:1719-1730, 2000.
30

The crystal structure of human AGT (pdb-ID 1EH6; Daniels *et al.*, EMBO J 19:1719, 2000) was resolved using a functional protein that was truncated after Asn207.
35

Summary of the invention

The invention relates to AGT mutants showing, when compared to the wild type human AGT, two or more advantageous properties selected from

- 5 (a) reduced DNA interaction;
- (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus;
- (c) improved expression yield as soluble protein and improved stability in various hosts;
- (d) improved stability under oxidising conditions;
- 10 (e) improved stability within cells after reaction with a substrate;
- (f) improved stability outside cells before and after reaction with a substrate;
- (g) improved *in vitro* solubility;
- (h) improved reactivity against O⁶-alkylguanine substrates;
- (i) reduced reactivity against DNA-based substrates; and
- 15 (j) reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates.

AGT mutants of the invention are mutants with the mentioned improved properties wherein between 1 and 25 amino acids of the wild type human AGT are substituted by other amino acids, and optionally 1 to 5 amino acids out of the continuous chain at one, two or three
20 positions are deleted or added and/or 1 to 4 amino acids at the N-terminus or 1 to 40 amino acids at the C-terminus are deleted.

The invention further relates to a method for detecting and/or manipulating a protein of interest wherein the protein of interest is incorporated into a fusion protein with the AGT
25 mutants of the invention. Another object of the invention are AGT fusion proteins comprising such AGT mutants and the protein of interest.

Detailed description of the invention

30 In the previously described method for detecting and/or manipulating a protein of interest, wherein the protein of interest is incorporated into an AGT fusion protein, the AGT fusion protein is contacted with particular AGT substrates carrying a label, and the AGT fusion protein is detected and optionally further manipulated using the label in a system designed for recognising and/or handling the label, the performance of AGT can be further improved
35 by replacing the wild type human AGT by mutant AGT. Such an improved method involving mutant AGT is the object of the invention. Another object are AGT mutants particularly suitable for the described method, and AGT fusion proteins comprising such AGT mutants

and one or more other proteins including at least one protein of interest. Protein of interest can be any protein.

AGT mutants of the invention comprise e.g. mutants which, when compared to the wild type human AGT or to known AGT mutants, show two or more properties selected from

- (a) reduced DNA interaction;
- (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus;
- (c) improved expression yield as soluble protein and improved stability in various hosts;
- (d) improved stability under oxidising conditions;
- (e) improved stability within cells after reaction with a substrate;
- (f) improved stability outside cells before and after reaction with a substrate;
- (g) improved *in vitro* solubility;
- (h) improved reactivity against O⁶-alkylguanine substrates;
- (i) reduced reactivity against DNA-based substrates; and
- (j) reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates.

(a) Reduced DNA interaction

A mutant AGT of the invention with "reduced DNA interaction" shows less than 20% of DNA binding, preferably less than 2% of DNA binding, most preferably no detectable DNA binding, when compared to wild type human AGT. The interaction with DNA is e.g. quantified by assessing the amount of copurified DNA from *E.coli* extracts under conditions of low salt and absence of DNase. This is compared between parallel purifications of fusion proteins (e.g. fusions to GST) of wild type (human) and mutant AGT by spectroscopic methods (ratio of absorbance at 260 and 280 nm). Wild type hAGT was shown to bind to DNA as a tetramer whereas the unbound hAGT protein stays monomeric (Rasimas *et al.*, J Biol Chem 278(10):7973-80, 2003). AGT mutants that are not able to bind to DNA also do not tetramerise.

(b) Localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus

A mutant AGT of the invention with "localisation no longer restricted to the nucleus" shows substantially uniform localisation of mutant AGT throughout the cell upon expression in eukaryotic cells, e.g. in mammalian cells. Subcellular localisation of AGT mutants is investigated by transiently transfecting AGT-deficient HeLa cells or CHO cells with a construct for constitutive expression under the human cytomegalovirus immediate early promoter. The cells are stained with cell membrane permeable diacetylfluorescein-modified

O⁶-benzylguanine (substance 4 of Juillerat *et al.*, Chem Biol 10:313-317, 2003) and analysed by confocal laser scanning microscopy. The fluorescence intensities for cytoplasm and nucleus are compared for mutant and wild type AGT. Wild type human AGT shows preferential localisation in the nucleus, and only marginal localisation in the cytoplasm.

5

(c) Improved expression yield as soluble protein and improved stability in various hosts

A mutant AGT of the invention with "improved expression yield as soluble protein", i.e. protein found in the soluble fraction after cell lysis and not in inclusion bodies, shows more than threefold expression yield, preferably more than fivefold expression yield, most preferably more than tenfold expression yield as soluble protein, when compared to wild type human AGT. This increased expression yield is at the same time a measure of "stability in the host" used for expression. Expression yield is measured in *E. coli* or in any other of the standard production cells for genetically modified proteins, e.g. yeast, or preferably insect cells, CHO cells or HeLa cells. For quantifying expression yield, AGT fusion proteins may be chosen such that the fusion partner allows easy purification and quantification. For example, expression yield in *E. coli* is determined by measuring and comparing the yield of soluble and insoluble-GST-wild-type-AGT fusion protein and GST-mutant-AGT-fusion protein from parallel *E. coli* expression cultures. Samples of the soluble fractions and insoluble fractions (i.e. the inclusion bodies) after cell lysis are subjected to SDS-PAGE, and the band staining intensities of the corresponding AGT fusion proteins are compared. Soluble protein is quantified after purification of the fusion protein from cell extracts by affinity chromatography (e.g. for GST-AGT fusion proteins by glutathione sepharose) by subjecting the purified fractions to a protein concentration assay (Bradford, Anal Biochem 72:248-54, 1976). The expression yield of similar proteins in the soluble fraction (difference in point mutations) can be used as a measure for protein stability in the unpurified as well as the purified state (Ohage *et al.*, J Mol Biol 291:1119-1128, 1999; Wirtz *et al.*, Protein Sci 8: 2245-50, 1999). Therefore, this is taken as a measure of folding stability and aggregation tendency.

(d) Improved stability under oxidising conditions

A mutant AGT of the invention with "improved stability under oxidising conditions" shows more than twofold yield of active protein, preferably more than fivefold yield of active protein, most preferably more than tenfold yield of active protein when compared to the wild type AGT or to known AGT mutants such as "PGEG-hAGT" (Juillerat *et al.*, Chem Biol 10:313-317, 2003), i.e. the AGT protein retains its activity towards AGT substrates after an incubation time of one or more hours under oxidising conditions in buffered aqueous solution (e.g. 100 mM NaCl, 10 mM HEPES, pH 7.4, no dithiothreitol or beta-mercaptoethanol added). The activities towards AGT substrates are measured after purification without

addition of reducing agents such as dithiothreitol or beta-mercaptoethanol. Alternatively, the activities of mutant AGT and wild type human are compared after their export into cellular compartments with oxidising redox potential (e.g. the periplasm of *E.coli*) due to their fusion to appropriate signal sequences. Activity towards AGT substrates under reducing and
5 oxidising conditions is compared by performing the reactions in the presence or absence of reducing agents such as dithiothreitol or beta-mercaptoethanol.

(e) Improved stability within cells after reaction with a substrate

A mutant AGT of the invention with "improved stability within cells" shows, after reaction with
10 a cell permeable substrate inside a cell (e.g. a mammalian cell), more than twofold stability, preferably more than threefold stability, most preferably more than sixfold stability, when compared to wild type human AGT or to known AGT mutants such as "PGEG-hAGT" (Juillerat *et al.*, Chem Biol 10:313-317, 2003). Stability is determined for a mutant AGT fusion protein after reaction with an AGT substrate by analysing the intensity and the
15 localisation of AGT fusion proteins with confocal laser scanning microscopy.

(f) Improved stability outside cells before and after reaction with a substrate

A mutant AGT of the invention with "improved stability outside cells" shows more than twofold stability, preferably more than fourfold stability, most preferably more than sixfold
20 stability, when compared to wild type human AGT or to known AGT mutants such as "PGEG-hAGT" (Juillerat *et al.*, Chem Biol 10:313-317, 2003). Stability before reaction is determined for the mutant AGT or for a mutant AGT fusion protein by incubating purified samples in buffered aqueous solution for up to two weeks at 4°C and up to six months at -20°C. At several time points, aliquots are taken and concentration of reactive AGT is estimated as
25 described by Juillerat *et al.*, Chem Biol 10:313-317, 2003. Stability for wild type AGT and the AGT mutant after reaction with a labelled substrate and after subsequent separation from unreacted substrate is determined by quantifying the concentration of the label in the soluble fraction over up to two weeks at 4°C and up to three months at -20°C.

(g) Improved in vitro solubility

A mutant AGT of the invention with "improved *in vitro* solubility" shows more than twofold solubility, preferably more than fivefold solubility, most preferably more than tenfold solubility, when compared to wild type human AGT. The *in vitro* solubility of wild type AGT and the AGT mutant is measured by determining the amount of protein that remains in the soluble
35 fraction after overnight incubation of purified samples at 4°C or up to 37°C at one or several concentrations established in a suitable buffer (e.g. 100 mM NaCl, 20 mM Tris, pH 8.0, 20% glycerol).

(h) Improved reactivity against O⁶-alkylguanine substrates

A mutant AGT of the invention with "improved reactivity" shows more than threefold reactivity, preferably more than fivefold reactivity, most preferably more than tenfold reactivity, when compared to wild type human AGT. Activity towards O⁶-benzylguanine substrates is measured as described by Juillerat *et al.*, Chem Biol 10:313-317, 2003.

(i) Reduced reactivity against DNA-based substrates

A mutant AGT of the invention with "reduced reactivity against DNA-based substrates" shows less than 10% reactivity against DNA-based substrates, preferably less than 1% reactivity against DNA-based substrates, most preferably no detectable reactivity against DNA-based substrates, when compared to wild type human AGT. The ability of wild type AGT or mutant AGT to react with alkylated DNA substrates is measured as the reaction of its inactivation by a synthetic oligonucleotide containing O⁶-benzyl guanine (SEQ ID NO:2, modified in position 14). Subsequently, the reactions are quenched by incubation with biotinylated O⁶-alkylguanine. Samples are subjected to Western blotting and detection with streptavidin derivatives to obtain kinetic constants for these substrates.

(j) Reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates

A mutant AGT of the invention with "reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates" shows less than 10% reactivity, preferably less than 2% reactivity, most preferably no detectable reactivity against N⁹-substituted O⁶-alkylguanine substrates, when compared to wild type human AGT or to known AGT mutants such as "PGEg-hAGT" (Juillerat *et al.*, Chem Biol 10:313-317, 2003). Such N⁹-substituted O⁶-alkylguanine substrates are the natural substrates of the wild type AGT. The ability of wild type AGT or mutant AGT to react with N⁹-modified O⁶-alkylguanine substrates is measured as the rate of its reaction with such substrates, e.g. with the low molecular weight N⁹-cyclopentyl-O⁶-benzylguanine in presence of biotinylated N⁹-unsubstituted O⁶-alkylguanine substrates in competition experiments. Subsequently, samples are subjected to Western blotting and detection with streptavidin derivatives to obtain kinetic constants for these substrates.

Preferred AGT mutants of the invention are those which have several preferred properties in common, e.g. those which show

(c) improved expression yield as soluble protein and improved stability in various hosts and
(h) improved reactivity against O⁶-alkylguanine substrates;
or

- (c) improved expression yield as soluble protein and improved stability in various hosts,
 - (d) improved stability under oxidising conditions,
 - (g) improved *in vitro* solubility, and
 - (h) improved reactivity against O⁶-alkylguanine substrates;
- 5 or
- (c) improved expression yield as soluble protein and improved stability in various hosts,
 - (d) improved stability under oxidising conditions,
 - (f) improved stability outside cells before and after reaction with a substrate,
 - (g) improved *in vitro* solubility, and
- 10 (h) improved reactivity against O⁶-alkylguanine substrates;
- or
- (a) reduced DNA interaction,
 - (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,
- 15 (c) improved expression yield as soluble protein and improved stability in various hosts,
- (h) improved reactivity against O⁶-alkylguanine substrates, and
 - (i) reduced reactivity against DNA-based-substrates;
- or
- (a) reduced DNA interaction,
- 20 (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,
- (c) improved expression yield as soluble protein and improved stability in various hosts,
 - (e) improved stability within cells after reaction with a substrate,
 - (h) improved reactivity against O⁶-alkylguanine substrates, and
- 25 (i) reduced reactivity against DNA-based substrates;
- or
- (a) reduced DNA interaction,
 - (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,
- 30 (c) improved expression yield as soluble protein and improved stability in various hosts,
- (h) improved reactivity against O⁶-alkylguanine substrates,
 - (i) reduced reactivity against DNA-based substrates, and
 - (j) reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates;
- or
- 35 (a) reduced DNA interaction,
- (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

- (c) improved expression yield as soluble protein and improved stability in various hosts,
- (e) improved stability within cells after reaction with a substrate,
- (h) improved reactivity against O⁶-alkylguanine substrates,
- (i) reduced reactivity against DNA-based substrates, and
- 5 (j) reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates;

More preferred AGT mutants are those which show

- 10 (c) more than fivefold expression yield as soluble protein and improved stability in various hosts and
- (h) improved reactivity against O⁶-alkylguanine substrates;
- or
- (c) more than fivefold expression yield as soluble protein and improved stability in various hosts,
- 15 (d) more than fivefold stability under oxidising conditions,
- (g) more than fivefold *in vitro* solubility, and
- ~~(h) more than fivefold reactivity against O⁶-alkylguanine substrates;~~
- or
- (c) more than fivefold expression yield as soluble protein and improved stability in various
- 20 hosts,
- (d) more than fivefold stability under oxidising conditions,
- (f) more than fourfold stability outside cells before and after reaction with a substrate,
- (g) more than fivefold *in vitro* solubility, and
- (h) improved reactivity against O⁶-alkylguanine substrates;
- 25 or
- (a) less than 2% of DNA binding,
- (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,
- (c) more than fivefold expression yield as soluble protein and improved stability in various
- 30 hosts,
- (h) more than fivefold reactivity against O⁶-alkylguanine substrates, and
- (i) less than 1% reactivity against DNA-based substrates;
- or
- (a) less than 2% of DNA binding,
- 35 (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

(c) more than fivefold expression yield as soluble protein and improved stability in various hosts,

(e) more than threefold stability within cells after reaction with a substrate,

(h) more than fivefold reactivity against O⁶-alkylguanine substrates, and

5 (i) less than 1% reactivity against DNA-based substrates;

or

(a) less than 2% of DNA binding,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

10 (c) more than fivefold expression yield as soluble protein and improved stability in various hosts,

(h) more than fivefold reactivity against O⁶-alkylguanine substrates,

(i) less than 1% reactivity against DNA-based substrates, and

(j) less than 2% reactivity against N⁹-substituted O⁶-alkylguanine substrates;

15 or

(a) less than 2% of DNA binding,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

20 (c) more than fivefold expression yield as soluble protein and improved stability in various hosts,

(e) more than threefold stability within cells after reaction with a substrate,

(h) more than fivefold reactivity against O⁶-alkylguanine substrates,

(i) less than 1% reactivity against DNA-based substrates, and

(j) less than 2% reactivity against N⁹-substituted O⁶-alkylguanine substrates;

25

Most preferred AGT mutants are those which show

(c) more than tenfold expression yield as soluble protein and improved stability in various hosts,

30 (d) more than tenfold stability under oxidising conditions,

(f) more than sixfold stability outside cells before and after reaction with a substrate,

(g) more than tenfold *in vitro* solubility, and

(h) more than tenfold reactivity against O⁶-alkylguanine substrates;

or

35 (a) no detectable DNA binding,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

- (c) more than tenfold expression yield as soluble protein and improved stability in various hosts,
(e) more than sixfold stability within cells after reaction with a substrate,
(h) more than tenfold reactivity against O⁶-alkylguanine substrates, and
5 (i) no detectable reactivity against DNA-based substrates;
or
(a) no detectable DNA binding,
(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,
10 (c) more than tenfold expression yield as soluble protein and improved stability in various hosts,
(e) more than sixfold stability within cells after reaction with a substrate,
(h) more than tenfold reactivity against O⁶-alkylguanine substrates,
(i) no detectable reactivity against DNA-based substrates, and
15 (j) no detectable reactivity against N⁹-substituted O⁶-alkylguanine substrates;
or
(a) reduced DNA-interaction;
(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,
20 (c) more than tenfold expression yield as soluble protein and improved stability in various hosts,
(d) more than tenfold stability under oxidising conditions,
(e) more than sixfold stability within cells after reaction with a substrate,
(f) more than sixfold stability outside cells before and after reaction with a substrate,
25 (g) more than tenfold *in vitro* solubility,
(h) more than tenfold reactivity against O⁶-alkylguanine substrates, and
(i) no detectable reactivity against DNA-based substrates;
or
(a) reduced DNA interaction,
30 (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,
(c) more than tenfold expression yield as soluble protein and improved stability in various hosts,
(d) more than tenfold stability under oxidising conditions,
35 (e) more than sixfold stability within cells after reaction with a substrate,
(f) more than sixfold stability outside cells before and after reaction with a substrate,
(g) more than tenfold *in vitro* solubility,

- (h) more than tenfold reactivity against O⁶-alkylguanine substrates,
- (i) no detectable reactivity against DNA-based substrates, and
- (j) no detectable reactivity against N⁹-substituted O⁶-alkylguanine substrates.

5 The AGT mutants of the state of the art only have some of the many desirable properties. For example the mutants described by Lim *et al.*, EMBO J 15:4050-4060, 1996 show reduced DNA binding and localisation in the cytoplasm on expression in mammalian cells. Mutants described by Juillerat *et al.*, Chem Biol 10:313-317, 2003, show increased reactivity towards O⁶-benzylguanine derivatives.

10

AGT mutants of the invention are mutants with the mentioned improved properties compared to wild type human AGT, wherein between 1 and 25, preferably between 2 and 15, in particular 2, 3, 4, 5 or 6 amino acids of the wild type human AGT are substituted by other amino acids, and optionally 1 to 5 amino acid out of the continuous chain at one, two or three
15 positions are deleted or added and/or 1 to 4 amino acids at the N-terminus or 1 to 40, preferably 20 to 35, in particular 25 to 30 amino acids at the C-terminus are deleted.

Preferably, amino acids in the following positions are replaced:

20 (A) Cys62, replaced by Ala or Val, preferably by Ala, which increases the expression yield in *E.coli* and renders the protein less susceptible to oxidation.

(B) Gln115-Gln116, replaced by Ala-Asn, Asn-Asn, Ser-His, Ser-Ser, Pro-Pro, Pro-Ser, Pro-Thr, or Thr-Ser, preferably by Ser-His, which allows increased expression in *E. coli* and
25 retains substrate reactivity comparable to wild type human AGT, in combination with other amino acid replacements, preferably replacement of Cys150-Ser151-Ser152, in particular replacement of Cys150.

(C) Lys125 replaced by Ala and Ala127-Arg128 replaced by Thr-Ala, which increases
30 expression yield in *E. coli*, reduces DNA binding and abolishes nuclear localisation in mammalian cells (Lim *et al.*, EMBO J 15:4050-4060, 1996), in combination with other amino acid replacements, preferably replacement of Gln115-Gln116 / Cys150-Ser151-Ser152 or replacement of Gly131-Gly132 / Met134-Arg135, in particular replacement of Cys62 / Gln115-Gln116 / Gly131-Gly132 / Met134-Arg135 / Cys150-Ser151-Ser152 and truncation
35 after 182.

(D) Gly131-Gly132 / Met134-Arg135 replaced by Val-His / Leu-Arg, Lys-Thr / Leu-Ser, Gln-Val / Leu-Ser, or Met-Thr / Met-Val, preferably Lys-Thr / Leu-Ser, or Gly131-Gly132 / Met134 replaced by Val-His / Leu, which increases expression yield in the periplasm and cytoplasm of *E.coli*, reduces DNA binding and abolishes reactivity with oligonucleotides (containing O⁶-alkyl-N⁹-deoxyribosylguanine), O⁶-alkyl-N⁹-deoxyribosylguanine and N⁹-cyclopentyl-O⁶-benzylguanine while increasing reactivity towards O⁶-alkylguanine substrates not substituted in the N⁹ position.

(E) Cys150-Ser151-Ser152 replaced by Asn-Ile-Asn, Pro-Leu-Pro, Pro-Arg-Thr, Ser-Phe-Pro-, or Ser-His-Thr-, preferably by Asn-Ile-Asn, or Cys150-Ser151 replaced by Phe-Asn or Arg-Asn, or Cys150 / Ser152 replaced by His / Thr, Leu / Asn, Leu / Asn, Leu / Pro or Pro / Leu, or Cys 150 replaced by Ser or Thr, which allows more efficient expression in the periplasm of *E. coli* compared to wild type hAGT, retains reactivity towards O⁶-alkylguanine substrates and renders the protein less sensible to oxidation and decreases DNA-binding.

(F) Pro140 / Asn157 / Ser159 replaced by Phe / Arg / Glu, or Pro140 / Asn157 / Gly160 replaced by Met / Trp / Val, or Asn157 / Ser159-Gly160-replaced by Gly / Glu-Ala, Gly / Asn-Trp, Pro / Gln-Cys or Gly-Gln-Trp, most preferably Gly-Glu-Ala, or Asn157 / Ser159 replaced by Gly / Glu (especially preferred), or Asn157 replaced by Gly or Arg in combination with other amino acid replacements, preferable with replacement of Gln115-Gln116 / Cys150-Ser151-Ser152 or replacement of Gly131-Gly132 / Met134-Arg135, in particular replacement of Cys62 / Gln115-Gln116 / Lys125 / Ala127-Arg128 / Gly131-Gly132 / Met134-Arg135 / Cys150-Ser151-Ser152 and truncation after 182, which increases the reaction rate towards O⁶-benzylguanine substrates not substituted in the N⁹ position (Juillerat *et al.*, Chem Biol 10:313-317, 2003) while increasing the expression yield in the periplasm and cytoplasm of *E.coli*, reducing DNA binding and abolishing reactivity with oligonucleotides (containing O⁶-alkyl-N⁹-deoxyribosylguanine), O⁶-alkyl-N⁹-deoxyribosylguanine and N⁹-cyclopentyl-O⁶-benzylguanine.

(G) Truncation after Gly182 (amino acids 183-207 deleted), which increases expression yield slightly.

Mutants of the invention are especially those wherein two or more, for example two, three or four out of the modifications (A) to (G) are present.

Particularly preferred are

mutant Cys62Ala, Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu, truncated after Gly182, which shows increased expression yield in *E.coli*, reduced sensitivity to oxidation, distribution throughout the cytoplasm in CHO cells, reduced DNA binding, and increased reactivity towards O⁶-benzylguanine substrates,

5

mutant Gly131Lys, Gly132Thr, Met134Leu, Arg135Ser, Asn157Gly, Ser159Glu, which shows increased expression yield in *E.coli*, at least 1000fold reduced DNA binding, increased reactivity towards O⁶-benzylguanine substrates, and substantially reduced reactivity towards O⁶-alkyl-N⁹-deoxyribosylguanine or N⁹-cyclopentyl-O⁶-benzylguanine,

10

mutant Gln115Ser, Gln116His, Cys150Asn, Ser151Ile, Ser152Asn, Asn157Gly, Ser159Glu, which shows substantially increased expression yield in *E.coli*, but retains activity towards O⁶-benzylguanine substrates, and

15

mutant Cys62Ala, Gln115Ser, Gln116His, Lys125Ala, Ala127Thr, Arg128Ala, Gly131Lys, Gly132Thr, Met134Leu, Arg135Ser, Cys150Asn, Ser151Ile, Ser152Asn, Asn157Gly, Ser159Glu, truncated after Gly182, which shows increased-expression-yield in *E.coli*, reduced sensitivity to oxidation, distribution throughout the cytoplasm in CHO cells, at least 1000fold reduced DNA binding, increased reactivity towards O⁶-benzylguanine substrates, and at least 100fold reduced reactivity towards O⁶-alkyl-N⁹-deoxyribosylguanine or N⁹-cyclopentyl-O⁶-benzylguanine.

20

Particularly preferred are the compound of the Examples.

25

Mutants are obtained by techniques well known to those skilled in the art. AGT variants may preferably be produced using protein engineering techniques known to the skilled person and/or using molecular evolution to generate and select new O⁶-alkylguanine-DNA alkyltransferases. Such techniques are e.g. site directed mutagenesis, saturation mutagenesis, error prone PCR to introduce variations anywhere in the sequence, and DNA

30

shuffling used after saturation mutagenesis. With the aid of the phage display method mutants are found with significantly increased activity towards O⁶-benzylguanine and AGT substrates of the invention and with increased stability under oxidising conditions. AGT can be functionally displayed as a fusion protein with the phage capsid protein pIII on filamentous phage, and the unusual mechanism of AGT can be used to select phages displaying AGT

35

with improved properties, e.g. increased reaction rate, see Juillerat *et al.*, Chem Biol 10:313-317, 2003.

In the present invention the protein or peptide of interest is fused to the AGT mutant described above. The protein or peptide of interest may be of any length and both with and without secondary, tertiary or quaternary structure, and preferably consists of at least twelve amino acids and up to 2000 amino acids. Examples of such protein or peptide of interest are provided below, and are e.g. enzymes, DNA-binding proteins, transcription regulating proteins, membrane proteins, nuclear receptor proteins, nuclear localization signal proteins, protein cofactors, small monomeric GTPases, ATP-binding cassette proteins, intracellular structural proteins, proteins with sequences responsible for targeting proteins to particular cellular compartments, proteins generally used as labels or affinity tags, and domains or subdomains of the aforementioned proteins. The protein or peptide of interest is preferably fused to the AGT mutant by way of a linker which may be cleaved by an enzyme, e.g. at the DNA stage by suitable restriction enzymes, e.g. AGATCT cleavable by *Bgl II*, and/or linkers cleavable by suitable enzymes at the protein stage, e.g. tobacco etch virus Nla (TEV) protease. Fusion proteins may be expressed in prokaryotic hosts, preferably *E. coli*, or eukaryotic hosts, e.g. yeast, insect or mammalian cells.

The AGT mutant has the property of transferring a label present on a suitable substrate described below to one of the cysteine residues of the AGT part of a fusion protein.

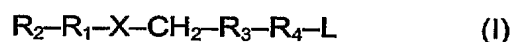
The fusion protein comprising protein of interest and the AGT mutant is contacted with a particular substrate having a label, as described below. Conditions of reaction are selected such that the AGT mutant reacts with the substrate and transfers the label of the substrate. Usual conditions are a buffer solution at around pH 7 at room temperature, e.g. around 25°C. However, it is understood that the AGT mutant reacts also under a variety of other conditions, and those conditions mentioned here are not limiting the scope of the invention.

The label part of the substrate can be chosen by those skilled in the art dependent on the application for which the fusion protein is intended. After contacting the fusion protein comprising AGT mutant with the substrate, the label is covalently bonded to the fusion protein. The labelled AGT mutant fusion protein is then further manipulated and/or detected by virtue of the transferred label. The label may consist of a plurality of same or different labels. If the substrate contains more than one label, the corresponding labelled AGT mutant fusion protein will also comprise more than one label which gives more options for further manipulating and/or detecting the labelled fusion protein.

Under "manipulation" any physical or chemical treatment is understood. For instance manipulation may mean isolation from cells, purification with standard purification

techniques, e.g. chromatography, reaction with chemical reagents or with the binding partner of a binding pair, in particular if the binding partner is fixed to a solid phase, and the like. Such manipulation may be dependent on the label L, and may occur in addition to "detection" of the labelled fusion protein. If the labelled fusion protein is both manipulated and detected,
5 detection may be before or after manipulation, or may occur during manipulation as defined herein.

The particular AGT substrates are those disclosed in patent application PCT/EP03/10889, e.g. compounds of formula (I)



wherein R_1-R_2 is a group recognized by AGT as a substrate;

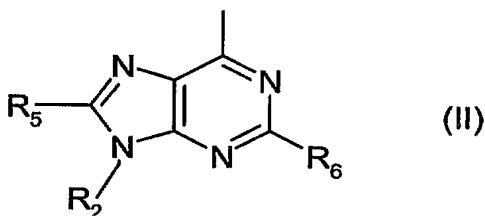
X is oxygen or sulfur;

15 R_3 is an aromatic or a heteroaromatic group, or an optionally substituted unsaturated alkyl, cycloalkyl or heterocyclyl group with the double bond connected to CH_2 ;

R_4 is a linker; and

L is a label, a plurality of same or different labels, a bond connecting R_4 to R_1 forming a cyclic substrate, or a further group $-R_3-CH_2-X-R_1-R_2$.

20 In a group R_1-R_2 , the residue R_1 is preferably a heteroaromatic group containing 1 to 5 nitrogen atoms, recognized by AGT as a substrate, preferably a purine radical of the formula (II)



25 wherein R_2 is hydrogen, alkyl of 1 to 10 carbon atoms, or a saccharide moiety;

R_5 is hydrogen, halogen, e.g. chloro or bromo, trifluoromethyl, or hydroxy; and

R_6 is hydrogen, hydroxy or unsubstituted or substituted amino.

30 If R_5 or R_6 is hydroxy, the purine radical is predominantly present in its tautomeric form wherein a nitrogen adjacent to the carbon atom bearing R_5 or R_6 carries a hydrogen atom,

the double bond between this nitrogen atom and the carbon atom bearing R₅ or R₆ is a single bond, and R₅ or R₆ is double bonded oxygen, respectively.

5 If R₆ is unsubstituted or substituted amino and the residue X connected to the bond of the purine radical is oxygen, the residue of formula (II) is a guanine derivative.

Other particular substrates useful in the method of the invention are compounds of formula (I) wherein R₁ is a purine radical of formula (II) and R₂ is cycloalkyl, e.g. cyclopentyl.

10 The present invention provides a method to label AGT mutant fusion proteins both *in vivo* as well as *in vitro*. The term *in vivo* labelling of a AGT mutant fusion protein includes labelling in all compartments of a cell as well as of AGT mutant fusion proteins pointing to the extracellular space. If the labelling of the AGT mutant fusion protein is done *in vivo* and the protein fused to the AGT mutant is a membrane protein, more specifically a plasma
15 membrane protein, the AGT part of the fusion protein can be attached to either side of the membrane, e.g. attached to the cytoplasmic or the extracellular side of the plasma membrane.

20 If the labelling is done *in vitro*, the labelling of the fusion protein can be either performed in cell extracts or with purified or enriched forms of the AGT mutant fusion protein.

If the labelling is done *in vivo* or in cell extracts, the labelling of the endogenous AGT of the host can be advantageously taken into account. If the endogenous AGT of the host does not accept O⁶-alkylguanine derivatives or related compounds as a substrate, the labelling of the
25 fusion protein is specific. In mammalian cells, e.g. in human, murine, or rat cells, labelling of endogenous AGT is possible. In those experiments where the simultaneous labelling of the endogenous AGT as well as of the AGT mutant fusion protein poses a problem, known AGT-deficient cell lines can be used.

30 When using a mutant AGT fusion protein that is non-reactive against a particular substrate in turn recognized by the endogenous AGT, such a non-reactive substrate can be used for blocking the activity of endogenous AGT before or while incubating the cells with a substrate designed to react with the particular mutant AGT fusion protein. For example, a mutant AGT may be used that does not react with N⁹-substituted O⁶-alkylguanine derivatives, e.g. with N⁹-
35 cyclopentyl-O⁶-benzylguanine. In mammalian cells containing endogenous AGT, this wild type AGT can then be blocked with N⁹-cyclopentyl-O⁶-benzylguanine prior to labeling or while

labeling a mutant AGT fusion protein with a different substrate recognized by this mutant AGT.

Examples

5

Abbreviations used:

DTT = 1,4-dithiothreitol

GST = glutathione-S-transferase (from *Schistosoma japonicum*)

HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid

10 IPTG = isopropyl β -D-1-thiogalactopyranoside

PEG = polyethylene glycol

PMSF = phenylmethanesulfonyl fluoride

SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis

15 Example 1: Mutations Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu

Two partially overlapping regions of the PGEG-hAGT gene, an AGT containing the mutations Asn157Gly, Ser159Glu (Juillerat *et al.*, Chem Biol 10:313-317, 2003), are amplified with the primers SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:4, SEQ ID NO:5 in separate reactions.

20 With respect to their partial complementarity, these two parts are assembled in a further PCR reaction, and amplified with the primers SEQ ID NO:3 and SEQ ID NO:4 to give rise to the complete gene now further containing the mutations Lys125Ala, Ala127Thr, Arg128Ala. The gene is subsequently cloned between the BamH1 and EcoR1 sites of the expression vector pGEX-2T (Pharmacia). This allows the expression of the inserted gene as a C-terminal
25 fusion to the GST protein, the gene of which is provided by the vector.

Protein expression from this vector is carried out in *E. coli* strain BL21. An exponentially growing culture is induced with 0.5 mM IPTG, and the expression is carried out for 3.5 h at 24°C.

30

Purification: The harvested cells are resuspended in a buffer containing 50 mM phosphate, 0.5 M NaCl, 1 mM DTT, supplemented with 1 mM PMSF and 2 μ g/mL aprotinin, and disrupted by lysozyme and sonification. The cell debris are separated by centrifugation at 40000 x g. The extract is applied to pre-equilibrated glutathione sepharose (Amersham)
35 which is then washed with 20 bed volumes (50 mM phosphate, 0.5 M NaCl, 1 mM DTT). The mutated GST-AGT fusion protein is eluted with 10 mM reduced glutathione in 50 mM

Tris-HCl pH 7.9. The purified protein is dialyzed against 50 mM HEPES pH 7.2; 1 mM DTT; 30 % glycerol and then stored at -80°C .

- Determination of protein yield: Purity and relative amounts of the GST-AGT fusion proteins are compared by running samples on SDS-PAGE. UV spectra of the pure fractions are recorded using a Perkin Elmer Lambda 10. For protein samples containing no significant amount of DNA, the extinction at 280 nm as a measure of protein content is compared to that of wild type hAGT or PGEG-hAGT that were purified under the same conditions.
- For comparing the DNA binding properties of the protein *in vitro*, the harvested cells are resuspended, and the GST fusion protein is purified following the same method but in a buffer containing 50 mM phosphate, 0.1 M NaCl, 1mM DTT. In the dialysed samples, the amount of subsequently co-purified DNA is estimated by UV spectroscopy. Spectra are recorded using a Perkin Elmer Lambda 10. The content of DNA corresponds to the ratio of the extinctions at 280 and 260 nm. This value is compared to the one obtained on purifying wild type hAGT or PGEG-hAGT, respectively.

- Activity assay: Purified mutated GST-AGT is incubated *in vitro* with a biotinylated O⁶-benzylguanine (substance 3a of Juillerat *et al.*, Chem Biol 10:313-317, 2003). In a total reaction volume of 80 μl , 0.2 μM GST-AGT are incubated with 1 μM substrate in 50 mM HEPES pH 7.2 and 1 mM DTT at room temperature. At several points of time an aliquot is quenched with 1 mM O⁶-benzylguanine (Sigma) in SDS-Laemmli buffer and subjected to Western blotting analysis (neutravidin-peroxidase conjugate (PIERCE), Renaissance reagent plus (NEN)). The intensity of the corresponding bands is quantified by a Kodak Image Station 440.

- In vivo* localization: The mutated AGT (Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu) gene is amplified with the primers SEQ ID NO:7, SEQ ID NO:8 and cloned between the NheI and BamHI sites of the vector pEGFP-Nuc (Clontech). This construct is transfected into CHO-cells deficient in endogenous AGT (B. Kaina *et al.*, Carcinogenesis 12, 1857-1867, 1991). After transient expression of the AGT during 24 h, the cells are incubated with 0.5 μM substance 4 of Juillerat *et al.*, Chem Biol 10:313-317, 2003 for 5 minutes and washed with PBS during 30 minutes. The cells are imaged by Laser scanning confocal microscopy using a 488 nm argon/krypton laser line on a Zeiss LSM 510 microscope (Carl Zeiss AG) with a water (1.2 numerical aperture) objective.

In *E.coli*, the GST fusion of mutant AGT Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu yields at least three times more soluble protein than the PGEG-hAGT. It shows at least ten times reduced DNA binding, and retains activity towards O⁶-benzylguanine substrates. In CHO cells, the fluorescently labeled mutated AGT is distributed throughout the cytoplasm, no preferential nuclear localization can be detected.

Example 2: Mutations Cys62Ala, Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu

The mutant AGT gene of Example 1 is amplified and cloned as described in Example 1 with the oligonucleotides SEQ ID NO:3, SEQ ID NO:10 and SEQ ID NO:4, SEQ ID NO:9 causing the further mutation Cys62Ala in the gene upon their incorporation by PCR. Protein expression, purification and determination of yield is carried out as described in Example 1. The mutant gene is PCR amplified with the primers SEQ ID NO:11, SEQ ID NO:12 that contain SfiI restriction sites to subclone the gene in fusion to the g3 protein of filamentous phage in the vector pAK100 (Krebber *et al.*, J Immunol Methods 201:35-55, 1997). When expressing the gene in the non-suppressor strain *E. coli* BL21, the amber stop codon terminates-translation-after-the AGT gene. Therefore, periplasmic-expression-of-mutant AGT protein from this vector is carried out as described in Example 1. The harvested cells are resuspended in a buffer containing 50 mM phosphate, 1 M NaCl, 1 mM DTT, supplemented with 1 mM PMSF and 2 µg/mL aprotinin and disrupted by lysozyme and sonification. The cell debris are separated by centrifugation at 40000 x g. The supernatant is directly subjected to quantification of protein yield via SDS-PAGE and activity assays as described in Example 1.

The mutant Cys62Ala, Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu retains activity towards O⁶-benzylguanine substrates. The yield of soluble GST fusion protein from this mutant AGT is at least two times higher than that of mutant Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu of Example 1. The yield of active AGT mutant from expression in the periplasm of *E.coli* is at least three times higher than that of PGEG-hAGT.

Example 3: Truncation at 182, Asn157Gly, Ser159Glu

The PGEG-hAGT gene (Asn157Gly, Ser159Glu, see Juillerat *et al.*, Chem Biol 10:313-317, 2003) is amplified with primers SEQ ID NO:3, SEQ ID NO:13 to introduce a stop-codon and a EcoRI site after codon 182, and subsequently cloned between the BamH1 and EcoR1 sites of the expression vector pGEX2T (Pharmacia). Protein expression, purification and estimation of expression yield is carried out as described in Example 1.

The truncated PGEG-hAGT mutant retains activity towards O⁶-benzylguanine substrates. The yield of soluble mutant GST-AGT fusion protein is at least two times higher than that of mutant PGEG-hAGT.

5

Example 4: Mutations Gly131Lys, Gly132Thr, Met134Leu, Arg135Ser, Asn157Gly, Ser159Glu

10 The PGEG-hAGT gene (Asn157Gly, Ser159Glu, see Juillerat *et al.*, Chem Biol 10:313-317, 2003) is PCR-amplified with the primers SEQ ID NO:11, SEQ ID NO:15 and SEQ ID NO:12, SEQ ID NO:14 and assembled as described in Example 1. The primers contain the nucleotide mixtures NNK (N=A, C, G or T; K= G or T) at positions corresponding to the codons 131, 132, 134, 135 of the hAGT gene. The gene is cloned in fusion to the g3 protein of filamentous phage in the vector pAK100 via SfiI restriction sites. The resulting gene library
15 is used for phage display.

Production of phages of this library is carried out in *E.coli* JM101 cells. An exponential culture is superinfected with helper phage and grown overnight at 24°C. The supernatant of this culture is incubated with 1 µM digoxigeninylated O⁶-benzylguanine (substance 2 of
20 Juillerat *et al.*, Chem Biol 10:313-317, 2003) for 6 minutes. In subsequent selection rounds, the reaction time is decreased to 90 seconds and 45 seconds, respectively, and the concentration of substrate is decreased to 10 nM to increase selection pressure. Phages are purified from this reaction by precipitation with 4% PEG / 3% NaCl. The phages carrying mutant AGT that is now covalently labeled with digoxigenin are isolated by incubation with
25 magnetic beads coated with anti-digoxigenin antibodies (Roche Diagnostics), and used for re-infection of bacteria.

Selected AGT mutants are subcloned into pGEX2T, expressed, purified and characterized as described in Example 1. Discrimination between N⁹ substituted and N⁹ unsubstituted O⁶-
30 benzylguanine substrates is evaluated by competition experiments for purified mutated GST-AGT *in vitro* with N⁹-substituted and N⁹-unsubstituted O⁶-benzylguanine. Aliquots of 0.2 µM GST-AGT are incubated with varying concentrations of N⁹ substituted substrate (0, 0.5, up to 100 µM) and 0.5 µM biotinylated O⁶-benzylguanine (substance 3a of Juillerat *et al.*, Chem Biol 10:313-317, 2003) in 50 mM HEPES pH 7.3 and 1 mM DTT at room temperature. After
35 45 min reactions are quenched by the addition of SDS-Laemmli buffer and heat denaturation. Samples are subjected to SDS-PAGE and Western blotting analysis (neutravidin-peroxidase

conjugate (PIERCE), Renaissance reagent plus (NEN)). The intensity of the corresponding bands is quantified by a Kodak Image Station 440.

5 The yield of soluble GST fusion protein from this mutant AGT is at least two times higher than that of PGEG-hAGT. The mutant AGT shows at least twofold further increased activity towards O⁶-benzylguanine substrates. Reactivity towards O⁶-alkyl-N⁹-deoxyribosylguanine or N⁹-cyclopentyl-O⁶-benzylguanine is reduced at least 10fold, for particular clones at least 100fold compared to PGEG-hAGT. Reactivity with oligonucleotides, e.g. the oligonucleotide SEQ ID NO:2 wherein guanine in position 14 carries an O⁶-benzyl group, is reduced at least 1000fold compared to PGEG-hAGT.

Example 5: Mutations Gln115Ser, Gln116His, Cys150Asn, Ser151Ile, Ser152Asn, Asn157Gly, Ser159Glu

15 A library of hAGT mutants is constructed as described in Example 4 from three PCR fragments using the primers combinations SEQ ID NO:11, SEQ ID NO:17; SEQ ID NO:16, SEQ ID NO:19 and SEQ ID NO:12, SEQ ID NO:18 in three separate PCR reactions. The gene is assembled from the partially overlapping fragments, two of them containing the randomized nucleotide mixtures NNK at positions corresponding to codons 115-116 and 20 150-152, respectively. Phage display selections are carried out as described in Juillerat *et al.*, Chem Biol 10:313-317, 2003. Selected proteins are subcloned into pGEX, expressed, purified and characterized as described in Example 1. Periplasmic expression is carried out as described in Example 2.

25 The yield of soluble GST fusion protein from this mutant AGT is at least two times higher than that of PGEG-hAGT. The mutant AGT retains activity towards O⁶-benzylguanine substrates. The yield of active AGT mutant from expression in the periplasm of *E.coli* is at least two times higher than that of PGEG-hAGT.

30 Example 6: Mutations Cys62Ala, Gln115Ser, Gln116His, Lys125Ala, Ala127Thr, Arg128Ala, Gly131Lys, Gly132Thr, Met134Leu, Arg135Ser, Cys150Asn, Ser151Ile, Ser152Asn, Asn157Gly, Ser159Glu, Truncation at 182

35 The mutations from Examples 2, 3, 4, 5 are combined: Four overlapping fragments of the mutant AGT gene (Cys62Ala, Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu) from Example 1 are amplified using the primer combinations SEQ ID NO:3, SEQ ID NO:23; SEQ ID NO:21, SEQ ID NO:22; SEQ ID NO:20, SEQ ID NO:25 and SEQ ID NO:24, SEQ ID

NO:13 assembled to the gene and subcloned into pGEX2T as described under Example 1. The mutant AGT is expressed and purified and characterized as described under Example 1.

- 5 The solubility of the AGT mutant or a mutant AGT fusion protein *in vitro* is measured by determining the amount of protein that remains in the soluble fraction after overnight incubation of purified samples at 4 or up to 37°C at one or several concentrations established in a suitable buffer (e.g. 100 mM NaCl, 20 mM Tris, pH 8.0, 20% Glycerol), and it is compared to that of wild type AGT or to known AGT mutants such as "PGEg-hAGT" (Juillerat *et al.*, Chem Biol 10:313-317, 2003).
- 10 The AGT containing the combined mutations of Examples 1-5 shows an expression rate further increased at least twofold compared to the mutants described in the Examples 1 through 5 and has an at least fourfold increased solubility *in vitro* when compared to wild type AGT. It retains the reactivity towards O⁶-benzylguanine, but shows significantly reduced
- 15 reactivity at least by a factor of 10 with N⁹-substituted O⁶-alkylguanine derivatives and reduced reactivity at least by a factor of 100 with DNA substrates.

Claims

1. An O⁶-alkylguanine-DNA alkyltransferase (AGT) mutant showing, when compared to the wild type human AGT, two or more advantageous properties selected from

- 5 (a) reduced DNA interaction;
- (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus;
- (c) improved expression yield as soluble protein and improved stability in various hosts;
- (d) improved stability under oxidising conditions;
- 10 (e) improved stability within cells after reaction with a substrate;
- (f) improved stability outside cells before and after reaction with a substrate;
- (g) improved *in vitro* solubility;
- (h) improved reactivity against O⁶-alkylguanine substrates;
- (i) reduced reactivity against DNA-based substrates; and
- 15 (j) reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates.

2. The AGT mutant according to claim 1 wherein the advantageous properties are

- (c) improved expression yield as soluble protein and improved stability in various hosts and
- (h) improved reactivity against O⁶-alkylguanine substrates;

20 or

- (c) improved expression yield as soluble protein and improved stability in various hosts,
- (d) improved stability under oxidising conditions,
- (g) improved *in vitro* solubility, and
- (h) improved reactivity against O⁶-alkylguanine substrates;

25 or

- (c) improved expression yield as soluble protein and improved stability in various hosts,
- (d) improved stability under oxidising conditions,
- (f) improved stability outside cells before and after reaction with a substrate,
- (g) improved *in vitro* solubility, and

30 (h) improved reactivity against O⁶-alkylguanine substrates;

or

- (a) reduced DNA interaction,
- (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

35 (c) improved expression yield as soluble protein and improved stability in various hosts,

- (h) improved reactivity against O⁶-alkylguanine substrates, and
- (i) reduced reactivity against DNA-based substrates;

or

(a) reduced DNA interaction,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

- 5 (c) improved expression yield as soluble protein and improved stability in various hosts,
(e) improved stability within cells after reaction with a substrate,
(h) improved reactivity against O⁶-alkylguanine substrates, and
(i) reduced reactivity against DNA-based substrates;

or

- 10 (a) reduced DNA interaction,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

(c) improved expression yield as soluble protein and improved stability in various hosts,
(h) improved reactivity against O⁶-alkylguanine substrates,

- 15 (i) reduced reactivity against DNA-based substrates, and
(j) reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates;

or

(a) reduced DNA interaction,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

- 20 (c) improved expression yield as soluble protein and improved stability in various hosts,
(e) improved stability within cells after reaction with a substrate,
(h) improved reactivity against O⁶-alkylguanine substrates,
(i) reduced reactivity against DNA-based substrates, and
25 (j) reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates;

3. The AGT mutant according to claim 1 or 2 wherein the advantageous properties are
(c) more than fivefold expression yield as soluble protein and improved stability in various hosts and

- 30 (h) improved reactivity against O⁶-alkylguanine substrates;

or

(c) more than fivefold expression yield as soluble protein and improved stability in various hosts,

(d) more than fivefold stability under oxidising conditions,

- 35 (g) more than fivefold *in vitro* solubility, and
(h) more than fivefold reactivity against O⁶-alkylguanine substrates;

or

(c) more than fivefold expression yield as soluble protein and improved stability in various hosts,

(d) more than fivefold stability under oxidising conditions,

(f) more than fourfold stability outside cells before and after reaction with a substrate,

5 (g) more than fivefold *in vitro* solubility, and

(h) improved reactivity against O⁶-alkylguanine substrates;

or

(a) less than 2% of DNA binding,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the

10 nucleus,

(c) more than fivefold expression yield as soluble protein and improved stability in various hosts,

(h) more than fivefold reactivity against O⁶-alkylguanine substrates, and

(i) less than 1% reactivity against DNA-based substrates;

15 or

(a) less than 2% of DNA binding,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

(c) more than fivefold expression yield as soluble protein and improved stability in various

20 hosts,

(e) more than threefold stability within cells after reaction with a substrate,

(h) more than fivefold reactivity against O⁶-alkylguanine substrates, and

(i) less than 1% reactivity against DNA-based substrates;

or

25 (a) less than 2% of DNA binding,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

(c) more than fivefold expression yield as soluble protein and improved stability in various hosts,

30 (h) more than fivefold reactivity against O⁶-alkylguanine substrates,

(i) less than 1% reactivity against DNA-based substrates, and

(j) less than 2% reactivity against N⁹-substituted O⁶-alkylguanine substrates;

or

(a) less than 2% of DNA binding,

35 (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

(c) more than fivefold expression yield as soluble protein and improved stability in various hosts,

(e) more than threefold stability within cells after reaction with a substrate,

(h) more than fivefold reactivity against O⁶-alkylguanine substrates,

5 (i) less than 1% reactivity against DNA-based substrates, and

(j) less than 2% reactivity against N⁹-substituted O⁶-alkylguanine substrates;

4. The AGT mutant according to claim 1 or 2 wherein the advantageous properties are

10 (c) more than tenfold expression yield as soluble protein and improved stability in various hosts,

(d) more than tenfold stability under oxidising conditions,

(f) more than sixfold stability outside cells before and after reaction with a substrate,

(g) more than tenfold *in vitro* solubility, and

(h) more than tenfold reactivity against O⁶-alkylguanine substrates;

15 or

(a) no detectable DNA binding,

~~(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,~~

20 (c) more than tenfold expression yield as soluble protein and improved stability in various hosts,

(e) more than sixfold stability within cells after reaction with a substrate,

(h) more than tenfold reactivity against O⁶-alkylguanine substrates, and

(i) no detectable reactivity against DNA-based substrates;

or

25 (a) no detectable DNA binding,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

(c) more than tenfold expression yield as soluble protein and improved stability in various hosts,

30 (e) more than sixfold stability within cells after reaction with a substrate,

(h) more than tenfold reactivity against O⁶-alkylguanine substrates,

(i) no detectable reactivity against DNA-based substrates, and

(j) no detectable reactivity against N⁹-substituted O⁶-alkylguanine substrates;

or

35 (a) reduced DNA interaction,

(b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,

- (c) more than tenfold expression yield as soluble protein and improved stability in various hosts,
 - (d) more than tenfold stability under oxidising conditions,
 - (e) more than sixfold stability within cells after reaction with a substrate,
 - 5 (f) more than sixfold stability outside cells before and after reaction with a substrate,
 - (g) more than tenfold *in vitro* solubility,
 - (h) more than tenfold reactivity against O⁶-alkylguanine substrates, and
 - (i) no detectable reactivity against DNA-based substrates;
 - or
 - 10 (a) reduced DNA interaction,
 - (b) localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus,
 - (c) more than tenfold expression yield as soluble protein and improved stability in various hosts,
 - 15 (d) more than tenfold stability under oxidising conditions,
 - (e) more than sixfold stability within cells after reaction with a substrate,
 - (f) more than sixfold stability outside cells before and after reaction with a substrate,
 - (g) more than tenfold *in vitro* solubility,
 - (h) more than tenfold reactivity against O⁶-alkylguanine substrates,
 - 20 (i) no detectable reactivity against DNA-based substrates, and
 - (j) no detectable reactivity against N⁹-substituted O⁶-alkylguanine substrates.
5. The AGT mutant according to any of claims 1 to 4 wherein between 1 and 25 amino acids of the wild type human AGT are substituted by other amino acids, and optionally 1 to 5 amino acid out of the continuous chain at one, two or three positions are deleted or added and/or 1 to 4 amino acids at the N-terminus or 1 to 40 amino acids at the C-terminus are deleted.
- 25
6. The AGT mutant according to claim 5 wherein two or more modifications are selected from
- 30 (A) Cys62 replacement by Ala or Val;
 - (B) Gln115-Gln116 replacement by Ala-Asn, Asn-Asn, Ser-His, Ser-Ser, Pro-Pro, Pro-Ser, Pro-Thr, or Thr-Ser;
 - (C) Lys125 replacement by Ala and Ala127-Arg128 replaced by Thr-Ala;
 - (D) Gly131-Gly132 / Met134-Arg135 replacement by Val-His / Leu-Arg, Lys-Thr / Leu-Ser,
 - 35 Gln-Val / Leu-Ser, or Met-Thr / Met-Val, or Gly131-Gly132 / Met134 replacement by Val-His / Leu;

(E) Cys150-Ser151-Ser152 replacement by Asn-Ile-Asn, Pro-Leu-Pro, Pro-Arg-Thr, Ser-Phe-Pro-, or Ser-His-Thr-, or Cys150-Ser151 replacement by Phe-Asn or Arg-Asn, or Cys150 / Ser152 replacement by His / Thr, Leu / Asn, Leu / Asn, Leu / Pro or Pro / Leu, or Cys 150 replacement by Ser or Thr;

- 5 (F) Pro140 / Asn157 / Ser159 replacement by Phe / Arg / Glu, or Pro140 / Asn157 / Gly160 replacement by Met / Trp / Val, or Asn157 / Ser159-Gly160 replacement by Gly / Glu-Ala, Gly / Asn-Trp, Pro / Gln-Cys or Gly-Gln-Trp, or Asn157 / Ser159 replacement by Gly / Glu, or Asn157 replacement by Gly or Arg; and
(G) truncation after Gly182.

10

7. The AGT mutant according to claim 5 wherein two or more modifications are selected from

(A) Cys62 replacement by Ala;

(B) Gln115-Gln116 replacement by Ser-His;

- 15 (C) Lys125 replacement by Ala and Ala127-Arg128 replaced by Thr-Ala;

(D) Gly131-Gly132 / Met134-Arg135 replacement by Lys-Thr / Leu-Ser, or Gly131-Gly132 / Met134-replacement by Val-His / Leu;

(E) Cys150-Ser151-Ser152 replacement by Asn-Ile-Asn, or Cys 150 replacement by Ser or Thr;

- 20 (F) or Asn157 / Ser159 replacement by Gly / Glu; and

(G) truncation after Gly182.

8. The AGT mutant according to claim 5 selected from mutants with modifications

- 25 Cys62Ala, Lys125Ala, Ala127Thr, Arg128Ala, Asn157Gly, Ser159Glu, truncated after Gly182;

Gly131Lys, Gly132Thr, Met134Leu, Arg135Ser, Asn157Gly, Ser159Glu;

- 30 Gln115Ser, Gln116His, Cys150Asn, Ser151Ile, Ser152Asn, Asn157Gly, Ser159Glu; and

Cys62Ala, Gln115Ser, Gln116His, Lys125Ala, Ala127Thr, Arg128Ala, Gly131Lys, Gly132Thr, Met134Leu, Arg135Ser, Cys150Asn, Ser151Ile, Ser152Asn, Asn157Gly, Ser159Glu, truncated after Gly182.

35

9. A method for detecting and/or manipulating a protein of interest wherein the protein of interest is incorporated into a fusion protein with an AGT mutant according to anyone of

claims 1 to 8, the AGT fusion protein is contacted with particular AGT substrates carrying a label, and the AGT fusion protein is detected and optionally further manipulated using the label in a system designed for recognising and/or handling the label.

- 5 10. An AGT fusion protein comprising an AGT mutant according to anyone of claims 1 to 8 and a protein of interest.

Abstract

The invention relates to AGT mutants showing, when compared to the wild type human AGT, two or more advantageous properties selected from (a) reduced DNA interaction; (b)

- 5 localisation of the expressed protein in eukaryotic cells that is no longer restricted to the nucleus; (c) improved expression yield as soluble protein and improved stability in various hosts; (d) improved stability under oxidising conditions; (e) improved stability within cells after reaction with a substrate; (f) improved stability outside cells before and after reaction with a substrate; (g) improved *in vitro* solubility; (h) improved reactivity against O⁶-alkylguanine
10 substrates; (i) reduced reactivity against DNA-based substrates; and (j) reduced reactivity against N⁹-substituted O⁶-alkylguanine substrates. Such AGT mutants with the mentioned improved properties are mutants wherein between 1 and 25 amino acids of the wild type human AGT are substituted by other amino acids, and optionally 1 to 5 amino acids out of the continuous chain at one, two or three positions are deleted or added and/or 1 to 4 amino
15 acids at the N-terminus or 1 to 40 amino acids at the C-terminus are deleted. The invention further relates to a method for detecting and/or manipulating a protein of interest wherein the protein of interest is incorporated into a fusion-protein with the AGT mutants of the invention.

Another object of the invention are AGT fusion proteins comprising such AGT mutants and the protein of interest.

Mutants.ST25
SEQUENCE LISTING

<110> Covalys Biosciences AG

<120> Mutants of O6-Alkylguanine-DNA Alkyltransferase

<130> P318

<160> 25

<170> PatentIn version 3.1

<210> 1

<211> 624

<212> DNA

<213> Homo sapiens

<400> 1	
atggacaagg attgtgaaat gaaacgcacc aactggaca gccctttggg gaagctggag	60
ctgtctgggt gtgagcaggg tctgcacgaa ataaagctcc tgggcaaggg gacgtctgca	120
gctgatgccg tggaggtccc agcccccgct gcggttctcg gaggtccgga gcccctgatg	180
cagtgcacag cctggctgaa tgcctatttc caccagcccg aggctatcga agagttcccc	240
gtgccggcac ttcaccatcc cgttttccag caagagtcgt tcaccagaca ggtgttatgg	300
aagctgctga aggttgtgaa attcggagaa gtgatttctt accagcaatt agcagccctg	360
gcaggcaacc ccaaagccgc gcgagcagtg ggaggagcaa tgagaggcaa tcctgtcccc	420
atcctcatcc cgtgccacag agtggctctgc agcagcggag cctgtgggcaa ctactccgga	480
ggactggccg tgaaggaatg gcttctggcc catgaaggcc accggttggg gaagccaggc	540
ttgggagggg gctcaggctt ggcaggggcc tggctcaagg gagcgggagc tacctcgggc	600
tccccgctg ctggccgaaa ctga	624

<210> 2

<211> 22

<212> DNA

<213> Artificial Sequence

Mutants.ST25

<220>

<223> substrate oligonucleotide containing O6-Benzylguanine at position
14

<400> 2
gtggtgggca gctgaggcgt gg

22

<210> 3

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> sense primer for cloning AGT into pGEX

<400> 3
cgaaatggat ccatggacaa ggattgtgaa atg

33

<210> 4

<211> 43

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for cloning AGT into pGEX

<400> 4
gcctttgaat tccgtctttg tagtcgtttc ggccagcagg cgg

43

<210> 5

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Sense primer for mutating K125A, T127A, R128A

<400> 5
gcaaccccgca agccacggca gcagtgggag g

31

<210> 6

<211> 31

<212> DNA

Mutants.ST25

<213> Artificial Sequence

<220>

<223> Antisense primer for mutating K125A, T127A, R128A

<400> 6
cctcccactg ctgccgtggc tgcgggggttg c 31

<210> 7

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> sense primer for cloning into eukaryotic pNUC vector

<400> 7
gatcgagcta gcgctaccgg tcgccaccat ggacaaggat tgtgaaatg 49

<210> 8

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for cloning into eukaryotic pNUC vector

<400> 8
gctagggatc ctacgtttcg gccagcaggc g 31

<210> 9

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> sense primer for mutating Cys 62 to Ala

<400> 9
gagcccctga tgcaggctac agcctggctg aatgc 35

<210> 10

Mutants.ST25

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for mutating Cys 62 to Ala

<400> 10
gcattcagcc aggctgtagc ctgcatcagg ggctc

35

<210> 11

<211> 60

<212> DNA

<213> Artificial Sequence

<220>

<223> Sense primer for cloning of AGT mutants into phage-display vector

~~<400> 11~~
ctactcgcg ccagccggc catggcggac tacaagaca tggacaagga ttgtgaaatg

60

<210> 12

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for cloning of AGT mutants into phage-display vector

<400> 12
ggaattcggc ccccgaggcc gcgtttcggc cagcaggcgg

40

<210> 13

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for cloning AGT truncated after 182 into pGEX

Mutants.ST25

<400> 13
gcctttgaat tccgtctttg tagtctccca agcctggctt cc

42

<210> 14

<211> 50

<212> DNA

<213> Artificial Sequence

<220>

<223> sense primer for randomisation codons 131, 132, 134, 135

<220>

<221> misc_feature

<222> (22)..(35)

<223> a or g or c or t

<220>

<221> misc_feature

<222> (22)..(35)

<223> n is a or g or c or t

<400> 14
cccaaagccg cgcgagcagt gnnknnkgca nnknnkggca atcctgtccc

50

<210> 15

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for randomisation codons 131, 132, 134, 135

<400> 15
tgctcgcgcg gctttggggg tgcctg

26

<210> 16

<211> 41

<212> DNA

<213> Artificial Sequence

Mutants.ST25

<220>

<223> Sense primer for randomisation codons 115-116

<220>

<221> misc_feature

<222> (19)..(23)

<223> n is a or g or c or t

<400> 16
ggagaagtga tttcttacnn bnnbtttagca gccctggcag g

41

<210> 17

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for randomisation codons 115-116

<400> 17
gtaagaaatc acttctccga atttcac

27

<210> 18

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Sense primer for randomisation codons 150-152

<220>

<221> misc_feature

<222> (19)..(26)

<223> n is a or g or c or t

<400> 18
ccgtgccaca gagtggtcnn bnnbnnbgga gccgtgggcg g

41

<210> 19

Mutants.ST25

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for randomisation codons 150-152

<400> 19

gaccactctg tggcacgg

18

<210> 20

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Sense primer for mutating G131K, G132T, M134L, R135S

<400> 20

gcagccacgg cagcagtga gacggcactg agtgg

35

<210> 21

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for mutating G131K, G132T, M134L, R135S

<400> 21

ggatagggac aggattgcc ctcagtgccg tcttcactgc

40

<210> 22

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Sense primer for mutating E115S, E116H

<400> 22

gtgaaattcg gagaagtgat ttcttactct cacttagcag c

41

Mutants.ST25

<210> 23

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for mutating E115S, E116H

<400> 23

cctgccaggg ctgctaagtg agagtaagaa atcac

35

<210> 24

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> sense primer for mutating C150N, S151I, S152N

<400> 24

cgtgccacag agtgggtcaat atcaatggag ccg

33

<210> 25

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense primer for mutating C150N, S151I, S152N

<400> 25

cgtaaccgcc cacggctcca ttgatattga cc

32